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Peptides and ATP binding cassette peptide transporters

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Abstract – In this review our knowledge of ATP binding cassette (ABC) transporters specific for peptides is discussed. Besides serving a role in nutrition of the cell, the systems participate in various signaling processes that allow (micro)organisms to monitor the local environment. In bacteria, these include regulation of gene expression, competence development, sporulation, DNA transfer by conjugation, chemotaxis, and virulence development, and the role of ABC transporters in each of these processes is discussed. Particular attention is paid to the specificity determinants of peptide receptors and transporters in relation to their structure and to the mechanisms of peptide binding. © 2001 Éditions scientifiques et médicales Elsevier SAS

peptide transport / signaling / ABC transporter

1. Introduction

Peptide transport systems not only play an important role in the nutrition of a cell, but, in microorganisms, they are also involved in various signaling processes. These include regulation of gene expression in proteolytic systems, competence development, sporulation, DNA transfer by conjugation, chemotaxis, and virulence development. Moreover, in pathogenic bacteria peptide transport systems are also involved in the defense against cationic antimicrobial peptides. The regulatory roles of the peptide transporters allow the bacteria to sense the local environment and adapt to these conditions by adjusting the expression of specific genes (for review see [13]). In many of these processes a specific peptide functions as signal molecule and ATP binding cassette (ABC) transporters can be involved in the internalization or excretion of this peptide.

ABC transporters are multicomponent systems that consist of two transmembrane domains and two ATP binding domains. In addition to these ubiquitous proteins, the systems involved in peptide uptake into the cell employ a specific ligand binding protein or receptor to capture the peptide. The individual proteins/domains can be expressed as separate polypeptides but they can also be fused to each other in any possible combination [24]. For instance, the oligopeptide transport system of *Lactococcus lactis*, Opp, consists of five separate proteins, whereas the glycine-betaine transport system of this organism, OpuA, consists of only two distinct polypeptides (*figure 1A*). One component of OpuA is the ATP binding protein, the other subunit consists of the receptor domain fused to the transmembrane part of the transporter. Each of these subunits is present twice in OpuA, implying that two ligand binding domains are present per functional complex [23]. The transmembrane domains/proteins of ABC transporters are highly hydrophobic and each normally consists of six membrane spanning segments. The ATP binding proteins are located at the cytoplasmic side of the membrane and fuel the transport process by hydrolyzing ATP. How and in which steps energy is coupled to the translocation process is still poorly understood.

The binding proteins of ABC transporters generally determine the substrate specificity of the system. In Gram-negative bacteria the receptors are located in the periplasm, while in Gram-positive bacteria they

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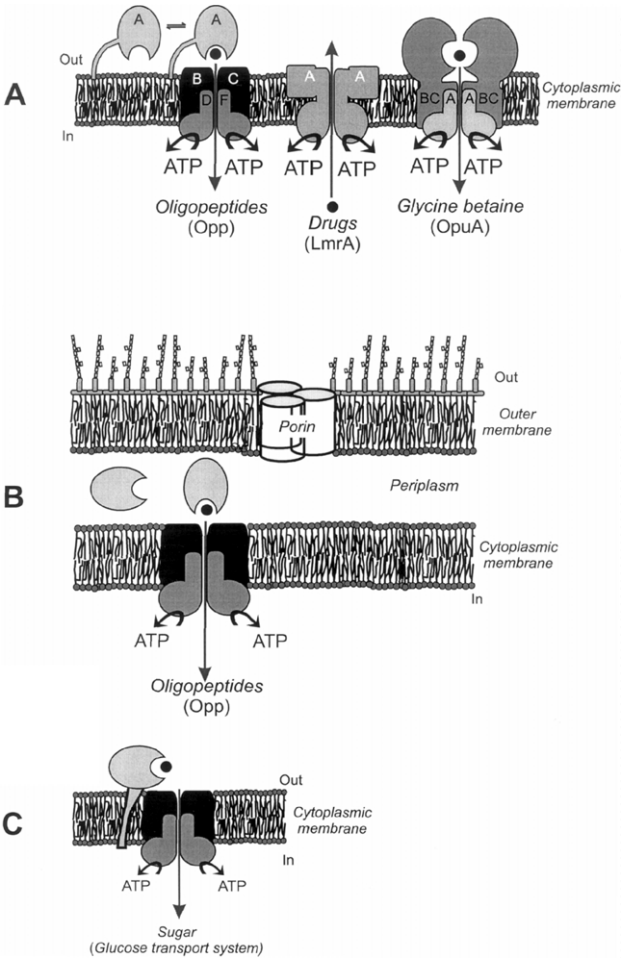

Figure 1. Domain organization of ABC transporters in Gram-positive (A) and Gram-negative (B) bacteria, and in archaea (C).

are anchored to the cell membrane via an N-terminal lipid moiety or fusion of the receptor to the translocator domain ([23], for reviews see [12, 24]). Binding proteins of the archaeon *Sulfolobus solfataricus* have been found to be attached to the cell membrane via a hydrophobic transmembrane segment ([2], *figure 1*). Whether this is a general feature of receptors of ABC transporters in archaea is not known. There is at least one case in which the situation is less clear. The trehalose/maltose binding protein (TMBP) of *Thermococcus litoralis* contains a predicted N-terminal signal sequence followed by a typical bacterial fatty acid attachment site. It is not known whether this attachment is indeed modified with a lipid anchor to associate the protein to the surface of the cell [25].

The binding mechanism of the receptor proteins of ABC transporters is generally thought to be according to the 'Venus fly-trap' mechanism, in which the binding site is formed by a cleft between two lobes that are connected by a flexible hinge. Upon binding of the ligand, the two lobes come together and enclose the ligand [46]. The binding of the peptide to the receptor domain is ATP-independent and exhibits hyperbolic saturation kinetics. How the receptor opens, after docking of a closed-liganded receptor onto the translocator, is unknown. For Opp of *L. lactis* it has been observed that the kinetics of the overall transport process is different for different substrates tested. Whereas class I peptides such as KYGK and AAAA exhibit normal Michaelis-Menten kinetics, the uptake of the majority of peptides (class II) increases sigmoidally with concentration. The different types of kinetic behavior have been explained by the presence of two or more ligand binding competent conformers of the receptor with different affinity for class I and II peptides [10].

Crystallographic analysis of the oligopeptide binding protein OppA of *Salmonella enterica* serovar *typhimurium* (*Salmonella typhimurium*) with and without ligand shows that the side-chains of the amino acids are accommodated in voluminous hydrated pockets. The water molecules act as flexible adapters, matching the hydrogen-bonding requirements of the protein and the ligand, and shielding charges on the buried ligand. The hydrogen bonds and salt bridges formed by the peptide backbone alone can drive binding and there is only a small contribution of the amino acid side chains to the binding affinity [51]. Although this binding mechanism is also observed for the dipeptide binding protein, DppA, of *Escherichia coli*, recent studies with OppA protein of *L. lactis* indicate that oligopeptide binding to this receptor is more complicated than suggested by the crystallographic studies. The differences and similarities in ligand binding to well-characterized peptide receptors are discussed in the next section.

In eukaryotes the ABC transporters are binding protein-independent as are the systems that expel rather than accumulate solutes. The best studied system with specificity for peptides is the human transporter associated with antigen processing (TAP). TAP is a heterodimer that consists of two homologous proteins, TAP1 and TAP2. Each monomer has ten instead of six predicted transmembrane α-helices and a nucleotide binding domain, and the peptide

binding site resides in transmembrane part formed by TAP1 and TAP2. This architecture differs from that of the binding-protein-dependent uptake systems, but also from other ABC type excretion systems. TAP transports peptides in the direction opposite that of binding-protein-dependent ABC transporters, that is, from the cytosol to the endoplasmatic reticulum (ER) lumen. This process is essential for the presentation of antigenic peptides by the major histocompatibility complex (MHC) to cytotoxic T lymphocytes (for review see [49]).

In this review, our understanding of the specificity determinants of peptide receptors and transporters in relation to their structure is discussed first. The roles of peptide binding and/or transport by ABC transporter(s) (components) in specific cellular processes such as proteolysis and nutrition, competence development, sporulation, chemotaxis and host defense mechanisms are discussed in the later sections.

2. Specificity determinants of peptide transport systems

The substrate specificity is known with greatest detail for human TAP and the Opp system of *L. lactis*. For both transport systems, combinatorial peptide libraries and fluorophore-labeled peptides have been used to determine size limits and side-chain preferences, and to characterize the binding pockets. In the case of TAP, the studies were performed with the complete system present in microsomal membranes [58], whereas, for Opp, the purified receptor protein OppA was used.

For binding protein-dependent transporters that can handle relatively large substrates (e.g., peptides composed of more than five to six residues), like the Opp system of *L. lactis*, the peptide is not likely to be totally enclosed by the binding protein. This notion can be made when one inspects the 3D structures of the homologous peptide binding proteins OppA and DppA from *S. typhimurium* and *E. coli*, respectively (*figure 2*). To study the binding mechanism of OppA from *L. lactis*, nonameric peptides were used in which the cysteine at position 1, 3, 4, 5, 6, 7, or 9 was selectively labeled with either bulky and nonfluorescent or bulky and fluorescent groups. These studies indicated that OppA encloses the first six residues of the peptide, whereas the remaining stick out and interact with the surface of the binding protein [30]. The amino

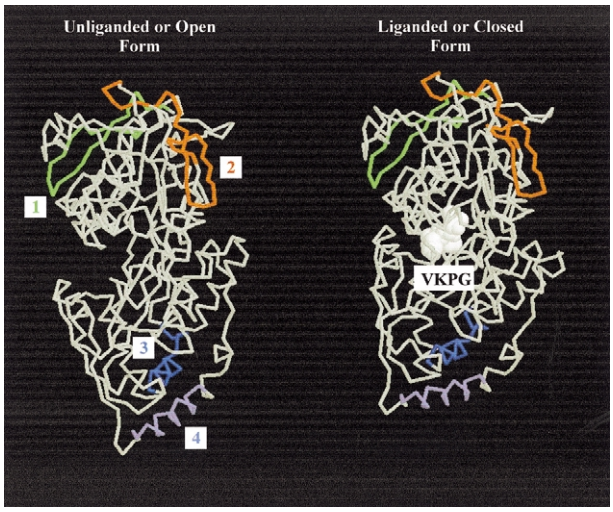

Figure 2. Projection of the four signature motifs of OppA homologues onto the 3-D structure of open/unliganded and closed/liganded OppA of *S. typhimurium*. The panel on the right shows the structure of OppA with bound tetrapeptide (VKPG).

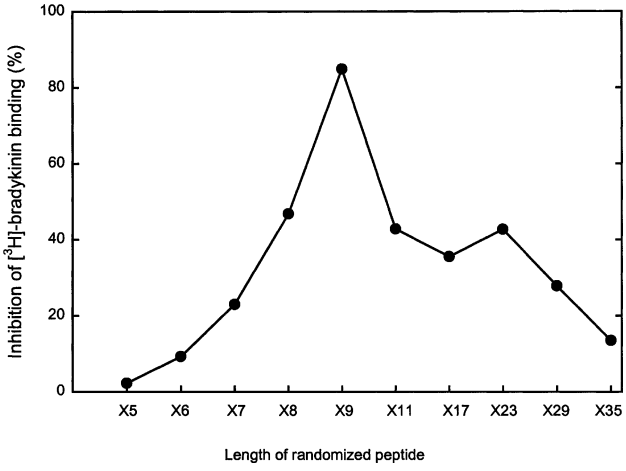

Figure 3. Length specificity of OppA. Fully randomized peptide libraries that differ in length from five to 35 residues were tested as competitors of the binding of the reporter peptide [^{3}H]-bradykinin to OppA [11].

acid residues of the substrate that are not enclosed by the protein contribute to the binding affinity of the peptide. In fact, the inhibition of binding of the reporter peptide bradykinin to $OppA_{Ll}$ increased more than 35 times when randomized nonameric peptides were tested instead of pentameric ones (*figure 3*). The combinatorial peptide libraries also showed that $OppA_{Ll}$ has a clear preference for hydrophobic and bulky amino acid residues at position 9 of nonameric

peptides. Such a preference would not be observed if the ninth residue was sticking out without interaction with the receptor surface. Next to position 9 in nonameric peptides, OppA of *L. lactis* also showed particular selectivity for residues at positions 4, 5 and 6, whereas the protein was more promiscuous at the other positions [11]. Although high resolution structural information is not available for OppA of *L. lactis*, the interactions of nonameric peptides, labeled with fluorescent or nonfluorescent groups of different size, show that the amino-terminal residues may be enclosed by the protein in a manner similar to that of $OppA_{St}$. The protein is restricted in the accommodation of bulky side-chains at positions 4, 5 and 6, which may prevent it from forming the closed liganded state.

The binding mechanism of OppA from *L. lactis* is also different from that of TAP. TAP is most selective at positions 1, 2, 3 and 9 of nonameric peptides, whereas for positions 4 to 8 hardly any side-chain contributions are observed. With regard to the peptide backbone, it was also found that the contact regions are confined to positions 1, 2, 3 and 9. Together with the strict requirement for free N- and C-termini, this leads to a model of peptide binding to TAP in which the peptide is fixed via the N-terminal residues (positions 1 to 3) and the C-terminal amino acid. The interactions are primarily via the peptide backbone but marked contributions are made by the side-chains at these positions. Residues 4 to 8 of a nonameric peptide could span a cavity with virtually no contact with TAP, allowing for longer peptides to be adopted, whereas peptides of less than eight residues are too short to span this distance. This model explains the fact that TAP has comparable affinity for peptides in the range of 8 to 16 residues [58].

Another difference, between OppA of *L. lactis* on the one hand and OppA and DppA of the enteric bacteria and human TAP on the other hand, is that $OppA_{Ll}$ does not require peptides with a free amino- or carboxyl-terminus. Acetylation of the N-terminal amino group or amidation of the C-terminal carboxyl group of the substrate only moderately lowers the affinity of OppA of *L. lactis* for the peptide [30].

The upper size limits for ligand binding to $OppA_{Ll}$ have not yet been defined, but the observation that libraries of 35 amino acid residues exhibit relatively high affinity suggests that any oligo- or polypeptide may bind. Since β-casein, a substrate of the proteolytic system of *L. lactis*, is a noncompact and largely flexible molecule with a high proportion of residues accessible to the solvent, it is even possible that OppA binds β-casein and thereby assists the proteinase PrtP in the breakdown of this molecule by retaining β-casein at the cell wall. Consistent with this hypothesis is the observation that OppA of *E. coli* exhibits chaperone properties. This chaperone activity was discovered in the refolding of urea-denatured citrate synthase and α-glucosidase in the presence and absence of periplasmic receptor proteins. In the presence of liganded as well as unliganded $OppA_{Ec}$, the yield of active renatured enzyme increased 3-fold compared with the refolding in the presence of BSA or in the absence of protein. The yield of renatured enzyme in the presence of OppA was comparable with the refolding in the presence of conventional chaperones such as DnaK or DnaJ. Since substrate loading of the receptor did not influence the interaction with unfolded proteins, it is thought that the peptide binding site in $OppA_{Ec}$ is not involved in the chaperone activity [47].

Finally, the Opp system of *L. lactis* transports peptides in the range of four up to at least 18 residues. When the kinetics of peptide binding to OppA and transport by Opp are compared, it becomes clear that the large differences in binding affinity (more than 1000-fold when particular penta- and nonameric peptides are compared) are not reflected in the affinity constants for uptake (only a 4-fold difference between penta- and nonameric peptides). This is rather unusual because for most binding protein-dependent transport systems, the K_m values for transport reflect the K_d values for substrate binding. There are three possible explanations for the observed differences in transport and binding affinity. Firstly, the differences could partly be methodological as it cannot be excluded that in the in vivo transport assays the peptide concentration, experienced by the binding protein, differs from the concentration in the solution due to adherence of peptides to the cell wall. Secondly, the effective concentration of OppA anchored to the cell membrane will be much higher than the concentrations used in the binding assays with purified OppA. It is possible that at high OppA concentration, two receptor proteins act cooperatively in the donation of ligand to the translocator. This suggestion is in line with the observation that the ABC transporter OpuA has two ligand binding domains per functional complex. Thirdly, depending on which step is rate-determining in the translocation process, the K_m for

transport may or may not reflect the K_d for binding [10, 29]. Further work is needed to discriminate between these possibilities.

3. Sequence comparison of oligopeptide binding proteins

The last decennium has seen an enormous increase in available sequence data. Several genomes have been analyzed, which has resulted in a large number of putative oligopeptide binding proteins. Using four conserved sequence patterns (*table I*), one of which was already described by Tam and Saier [53], more than 150 putative oligopeptide binding proteins from various bacterial and archaeal species were retrieved. Details will be described in a forthcoming paper. About 50% of the sequences have the aspartate that, in the structural analysis of OppA of *S. typhimurium* and DppA of *E. coli*, has been recognized as the residue that forms a salt bridge with the N-terminal α-amino group of the bound peptide. *Figure 4* shows part of the alignment of some of the receptor proteins with the conserved aspartate. Since the substrate specificity of peptide receptors is best documented for $OppA_{Ll}$, we also included this protein even though it lacks the aspartate and seems quite distantly related to the other sequences. Two alternative residues in the sequence of OppA of *L. lactis*, Asp^{487} or Asn^{486}, could fulfil the role of interacting with the N-terminal residue. The Asn^{486} could do so by forming a hydrogen bound with the N-terminal residues, which is in line with the observation that acetylation does not have such a profound effect on binding in $OppA_{Ll}$ as observed with other receptor proteins [17, 30, 41–43, 48].

Among the putative oligopeptide binding proteins that do not contain the N-terminal amino group accepting aspartate, there are several that have a glutamate residue at this position. In the other cases, the aspartate is replaced by alanine, glycine, leucine, valine, arginine, tyrosine or phenylalanine. This variation in residues might reflect the fact that several of these binding proteins do not bind peptides. For instance, heme is bound by HbpA of *Haemophilus influenzae* [20], agrocinopines by AccA of *Agrobacterium tumefaciens* [22], and nickel by NikA of *E. coli* (*table II*, [36]).

The positively charged residues, implicated in the formation of a Coulomb interaction with the C-terminal carboxyl group of bound di-, tri-, tetra- or pentapeptides and identified in the 3-D structure of OppA from *S. typhimurium* or DppA from *E. coli* [14, 38, 51, 54–56], are not conserved. This suggests that the typical binding characteristics of DppA from *E. coli* and OppA from *S. typhimurium* are not a general property of the family, not even in the members known to bind peptides. For instance, the carboxyl-terminal binding residues are not conserved in the well-characterized dipeptide binding protein DppA of *L. lactis* [48].

It is illustrative that the most highly conserved regions are not located around the residues that are involved in peptide binding or formation of the hydrated pockets in DppA from *E. coli* and OppA from *S. typhimurium*. Projection of the conserved

Table I. Conserved patterns observed in oligopeptide receptor proteins.

Pattern 1 (red)[a]
(OppA_Salty: 83–110)
(DppA_Ecoli: 76–104)
(OppA_Lacla: 106–133)
[GP]-X-[AILMV]-A-X_2-[ILVWY]-X_2-[DKNST]-$X_{0,1}$-[DEGNS]-[AGNS]-$X_{2,3}$-[AFILVWY]-X-[FILVY]-X-[ILV]-[KR]-X_4-[FW]-$X_{1,3}$-[DENQ]-G
Pattern 2 (green)
(OppA_Salty: 214–236)
(DppA_Ecoli: 208–233)
(OppA_Lacla: 241–266)
[ILMV]-X-[NST]-G-[AP]-[FWY]-X-[FILMPV]-X_8-[AILMVY]-X_4-[MNS]-X_2-[FWY]-[FWY]
Pattern 3 (blue)
(OppA_Salty: 314–327)
(DppA_Ecoli: 309–322)
(OppA_Lacla: 354–367)
[FILMV]-R-[EHKQR]-[AS]-[FILMV]-X_2-[GAST(ilmv)]-[FILMV]-[DN]-[HKNQR]-X_2-[FILMVY]
Pattern 4 (purple)
(OppA_Salty: 365–378)
(DppA_Ecoli: 354–368)
(OppA_Lacla: 401–415)
[FQWY]-[DN]-[LP]-[ADEKNQ]-$X_{0,9}$-[EKNQR]-[AS]-[AKNQR]-[AEKQRS]-[AEILMQV]-[ILMV]-[ADEKNQS]-[ADEKNQS]-[ADILM]-G-[FILMVWY]

[a] Pattern 1 is an extended version of the pattern described by Tam and Saier [51].

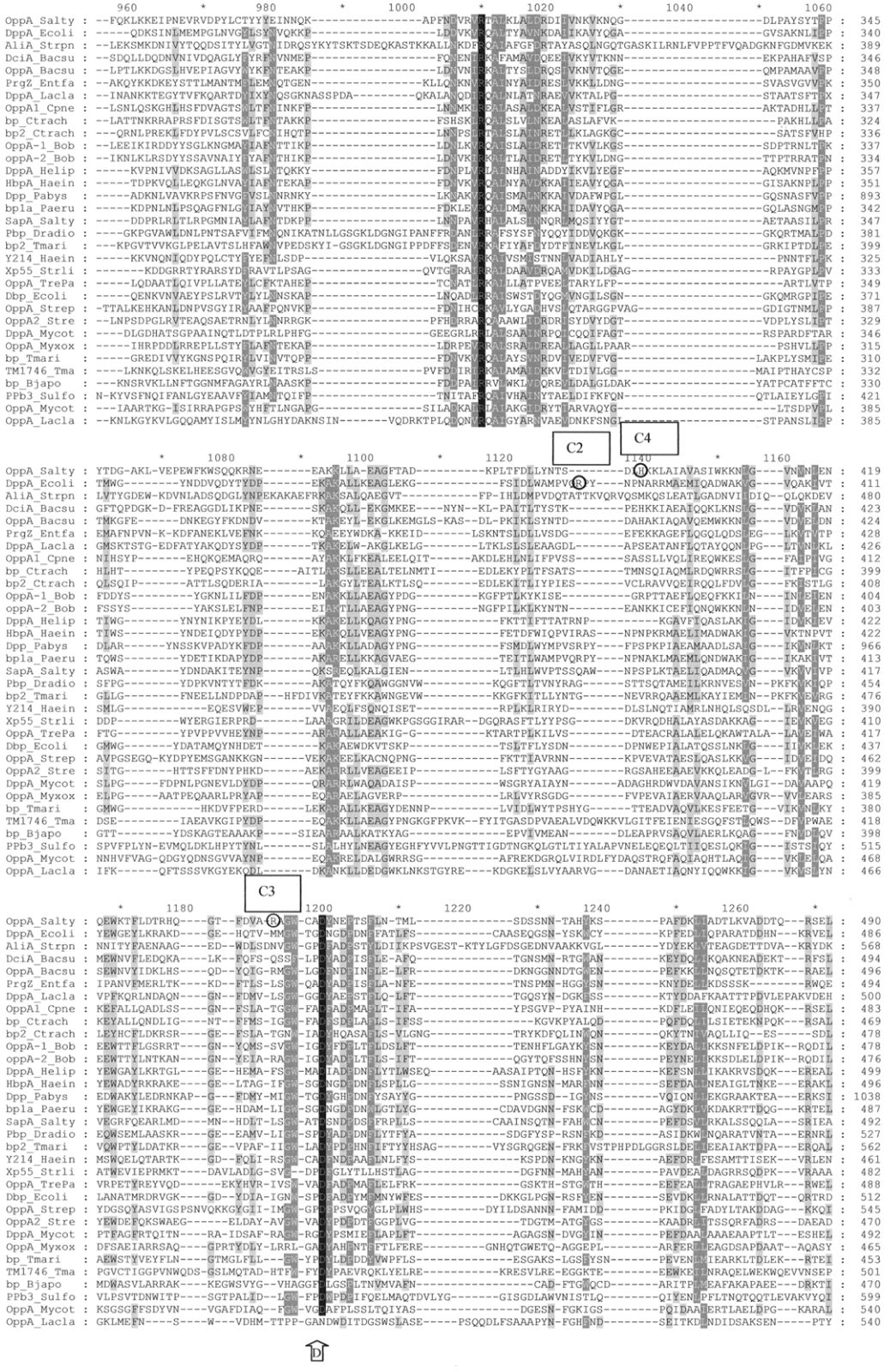
C2
C4
C3

Figure 4. Part of the sequence alignment of oligopeptide binding proteins. Sequences were retrieved from databases using Blast [3], PSI-Blast [4] and PHI-Blast [57] software. Sequences were aligned using ClustalX software [55]. Only 33 of the more than 150 sequences are shown. Arrow with D inside refers to the aspartate that interacts with N-termini of peptides as revealed by X-ray analysis of liganded $OppA_{St}$ and Dpp_{Ec} crystals. C2, C3 and C4 refer to the residues that interact with carboxyl-termini of di-, tri- and tetrapeptides, respectively.

Table II. Specificity of OppA and homologues.

Receptor protein	Substrate
OppA Salty	tri-, tetra- and pentapeptides
DppA Ecoli	dipeptide
Ali-group	oligopeptides
Hpp-group	hexa- and heptapeptides
OppA_Strepn	not studied
DciAE_Bacsu	> dipeptide
DppA_Trepa	not studied
OppA_Bacsu	tri, tetra- and pentapeptides
OppA's_Cpneu	not studied
PrgZ_Entfa	conjugative peptide (heptapeptide)
TraC_Entfa	conjugative peptide (octapeptide)
SbpA_Llac	not studied
DppA_Llac	di- and tripeptides
OppA_Ecoli	tri-, tetra- and pentapeptides
OppA's_Haein	not studied
MppA_Ecoli	murein
OppA_Vchlo	not studied
OppA's_Bor's	plasminogen (polypeptide)
DppA's_Helip	not studied
DppA_Bacfi	not studied
HbpA_Haein	heme
SapA_Erchy	antimicrobial peptides
SapA_Salty	antimicrobial peptides
SapA_Haein	not studied
Bp'_Campj	not studied
Acca_Argtu	agrocinopine
Y214_Haein	not studied
Orf4_Metba	not studied
OppA_apern	not studied
DppA_orcho	not studied
Dbp_Ecoli	not studied
OppA's_Mycot	not studied
OppA_Myxox	not studied
Pbb3 Sulfo	not studied
OppA Llac	not studied

sequence motifs on the known 3-D structure of $OppA_{St}$ (*figure 2*) shows that these regions are located on the receptor surface. In general, surface residues have a large freedom of mutation, except in the situation were they are important for interaction with other proteins. This could be an indication that the conserved surface located regions are actually involved in docking of the loaded receptors onto the membrane components of Opp.

If one considers the size-variation of the oligopeptides bound by the various binding proteins, it is clear that total enclosure of the ligands is sometimes impossible. Studies with OppA of *L. lactis* have clearly shown that binding of larger ligands is allowed, and that areas of the protein other than the cleft alone are involved in the binding of the peptide-ligand [11, 30]. The charge neutralization of the C-terminal carboxyl of the peptide by a cationic residue in the protein may be an exception in terms of binding mechanism. It seems more common that peptides larger than 5 residues can be bound and, therefore, that only part of the peptide is enclosed by the receptor. In this regard, the binding of human plasminogen by the OppAs of the *Borellia* genus is relevant to note [26]. This observation is consistent with the reports on chaperone activity ascribed to several receptor proteins associated with ABC transporters [47].

4. Physiological role of peptide transport

4.1. Source of nitrogen and metabolic energy

The primary function of the oligopeptide transport systems (Opp) in bacteria lies in the uptake of breakdown products of exogenous proteins and/or in the recycling of cell-wall peptides. For Opp of *E. coli*, it has been shown that the system is involved in the recycling of cell wall peptides. Later studies by Park and coworkers showed that another peptide binding protein, MppA, was essential for the transport of murein tripeptide. This binding protein is a homologue of OppA with 46% identical residues.

For the transport of the murein peptide, MppA uses the membrane-bound components of the Opp system. The second bond in murein tripeptide is an amide between the γ-carboxyl of D-glutamyl and the L-amino group of meso-diaminopimelate. Growth experiments revealed that MppA is necessary for growth on murein peptide, whereas mutants with wild-type level MppA but without OppA are unable to grow on normal α-linked tripeptides [40].

Lactic acid bacteria are multiple amino acid auxotrophs that require the uptake of either amino acids or peptides for their growth. For these organisms, exogenous proteins such as caseins represent an important source of nitrogen. In case of *L. lactis* it has been shown that β-casein is degraded to peptides of five to 30 amino acid residues, and a substantial fraction of these is imported into the cell via Opp. It has been shown that Opp of *L. lactis* accepts peptides in the range of five to ten residues when it is supplied with a complex mixture of β-casein-derived peptides [28]. This surprising finding was corroborated later on in studies with individual peptides of various lengths, where it was shown that peptides up to at least 18 residues are transported [10]. Inside the cell, the peptides are broken down by an array of peptidases to make the amino acids available.

In addition to Opp, *L. lactis* possesses at least two other peptide transport systems, that is the ABC transporter Dpp and the proton motive force-driven transporter DtpT. Both are specific for di- and tripeptides, and these systems thus do not have a direct nutritional role in *L. lactis* as di- and tripeptides are not formed in significant amounts in the breakdown of β-casein. By making single, double and triple peptide transport mutants of *L. lactis*, it has become clear that DtpT and Dpp participate indirectly in nitrogen metabolism as internalized di- and tripeptides affect the expression of various components of the proteolytic system, that is, the proteinase PrtP, the peptidases PepN and PepC, and different peptide transport systems. The regulation of expression of the proteolytic enzymes is achieved via, for example, the uptake of di-valine, which causes a 30–70% lowering of the protein levels. This repressing effect is no longer observed in the presence of high concentrations of valine, indicating that accumulation of di-peptide is required for the repressing effect (Sanz et al., submitted for publication). For both Gram-negative and Gram-positive bacteria, it has been observed that the expression of the *dpp* operon is regulated by nutrients present in the medium. The expression of the Dpp in *Bacillus subtilis* is repressed by amino acids and rapidly metabolizable carbon sources like glucose. During conditions of nutrient limitation, like in the early stationary phase and at the onset of sporulation, the Dpp system is maximally expressed [50]. The *dpp* operon of *E. coli* is subject to nitrogen and not carbon regulation. In group A streptococcal strains, dipeptides as sole source of essential amino acids increase the expression of *Dpp*, but the effect is not the same for all components. The ratio of DppA (receptor protein) and DppBCDE (other components of the transporter) decreases in the presence of dipeptides [45]. It is possible that the relative overexpression of the receptor protein serves to capture the peptides more efficiently when the concentration in the medium is low.

4.2. Competence and sporulation

Competent cells express a unique set of genes whose products allow the cells to import DNA fragments from the external medium. The competent cells bind double-stranded DNA, after which one strand is degraded. Fragments of the complementary strand are imported into the cell and recombination can take place with homologous regions in the DNA of the recipient. This process plays a role in the evolution of species and mediates a rapid mixing of alleles. In this way, for example, bacteria assembled a mosaic set of different antibiotic resistance genes, resulting in species that are resistant to different types of antibiotics. Competence is not a constitutive trait, but regulated by a quorum-sensing pheromone signal to ensure that cells only become competent when they are likely to encounter DNA that is released by lysis of other cells in the population [13]. In *B. subtilis*, competence typically develops in only 1–10% of the cells of a culture. Adding more competence pheromone to the culture does not increase the recombination frequency, suggesting that some other signal is limiting, or that there is a built-in mechanism to limit the size of the competent population [33].

The competence stimulating peptide has been identified for a few microorganisms. In *Streptococcus pneumoniae*, the pheromone signal is derived from a peptide, which is the product of the *comC* gene. The 41 amino-acids-long prepeptide contains an N-terminal double-glycine consensus processing site that is also found in peptide bacteriocins. This peptide is exported by a specialized ABC transporter, encoded by *comAB*, that cleaves off the signal sequence

prior to the translocation of the mature peptide. The proteolytic domain of the receptor is located at the cytoplasmic face of the membrane. The mature peptide is 17 amino acids long, and analysis of the structural requirements of the peptide showed that only seven residues are essential for its function. Two charged residues at the termini and five hydrophobic residues in the middle of the peptide are obligatory; all the other residues could be replaced. The peptide is not transported into the target cell but binds to a histidine kinase receptor (*comD*) that forms part of a two-component regulator system. The receptor phosphorylates the response regulator (*comE*) and this transcription factor causes the upregulation of *comCDE* and other operons involved in the development of competence [21].

In *B. subtilis*, there are two signal peptides known to be involved in competence: (i) ComX, a peptide of nine or ten amino acid residues that enhances competence; and (ii) competence stimulating factor (CSF), a pentameric peptide with the sequence ERGMT and derived from a precursor of 40 amino acid residues. Besides a role in competence development, CSF is also involved in the regulation of sporulation. The ComX peptide interacts with a 2-component regulator system in a manner comparable to that described for ComCDE of *S. pneumoniae*. CSF, on the other hand, is transported via Opp (encoded by *spo0K*) and functions intracellularly. When CSF is expressed inside the cell in the mature form, the competence response is comparable to that in the wild-type situation. CSF has different effects depending on the concentration in the cell. At low concentration (1–10 nM), CSF stimulates the expression of genes required for competence, whereas at high concentration (20 nM–1 μM) the effect of the peptide is twofold. It inhibits the expression of genes involved in competence development and, at the same time, the peptide stimulates the expression of genes required for sporulation. Each amino acid residue of CSF peptide is important for competence development, because single alanine substitutions result in an inactive peptide. For the sporulation response, on the other hand, the glycine can be replaced by alanine, resulting in a four times stimulated response compared to ERGMT [31]. Inside the cell, the peptides interact with a phosphorelay signal transduction pathway that controls both competence and sporulation. This pathway includes a kinase (KinA), a response regulator (Spo0F), a protein phosphatase (RapA), and a transcriptional regulator (Spo0A) that activates sporulation [44].

Similar to ComX, which is specific for competence development, PhrA, a pentameric peptide (ARNQT), derived from a 44 amino acid prepeptide, is specifically involved in the stimulation of sporulation. PhrA functions inside the cell, like CSF, and is transported via the Opp system. The peptide functions as effector of a transcription regulator in the phophorelay signal transduction pathway that controls competence/sporulation.

The mechanism of processing and secretion of the precursors of CSF and PhrA is not known, but recent work of Kobayashi and coworkers shows that a functional Sec translocation machinery is necessary for sporulation in *B. subtilis*. However, addition of PhrA to the medium of *sec*A12 mutants did not restore the sporulation response in this strain. This suggests that additional steps in sporulation in *B. subtilis*, e.g., the excretion of another peptide, also require a functional Sec translocase [27].

4.3. Conjugation

Conjugation is the transfer of certain plasmids from donor cells in response to signals (sex pheromones) produced and excreted by the recipient cells. Pheromones are small hydrophobic peptides of seven or eight amino acids that are transported into donor cells via the membrane components of Opp. Pheromone binding occurs by a specific receptor protein that is homologues to the general peptide receptors named OppA. The pheromone (cCF10), carried by the pCF10 conjugative plasmid of *Enterococcus faecalis*, is bound by PrgZ. A striking difference between PrgZ of *E. faecalis* and OppA of *L. lactis*, *E. coli* or *S. typhimurium* is the high affinity of the pheromone receptor for LVTLVFV. This ensures a high specificity and enables the cells to elicit a specific response with only 1–10 pM of the pheromone in the medium. In mutants without PrgZ, but with a functional Opp system, signaling still occurs but higher concentrations of the pheromone are necessary. This indicates that OppA is able to bind the pheromone but with lower affinity than PrgZ. In strains lacking OppD the pheromone response is no longer observed, indicating that transport of the pheromone into the cell is obligatory and takes place via the membrane components of Opp [32]. Another pheromone binding protein, TraC, is involved in the

Table III. Overview of conjugative plasmids of *E. faecalis* with corresponding pheromones and inhibitors.

Plasmid	Pheromone/inhibitor	Prepheromone/inhibitor[a] (peptide indicated in bold)	Inhibition of [^{3}H] cPD1 binding to TraC (IC_{50} [nM])[b]
pAD1	cAD1 (8-mer)[c]	MKVNKFVKGFAAIA**LFSLVLAG**	> 1000
	iAD1 (8-mer)[c]	MSKRAMKKIIPLIT**LFVVTLVG**	> 1000
pAM373	cAM373 (7-mer)	MLKKPFLLFFSLLG**AIFILAS**	> 1000
	iAM373	–	> 1000
pCF10	cCF10 (7-mer)	VKKYKRLLLMAG**LVTLVFV**	126
	iCF10 (7-mer)	MKTTLKKLSRYIAVVI**AITLIFI**	100
pOB1	cOB1 (8-mer)	MKKRTLWSVITVA**VAVLVLGA**	> 1000
	iOB1 (8-mer)	(precursor unknown) **SLTLILSA**	16.5
pPD1	cPD1 (8-mer)	MRKLNRWLYGSGLL**FLVMFLSG**	34.2
	iPD1 (8-mer)	MKQQKKHIAALLF**ALILTLVS**	78.5

[a] Data from [9]. [b] Data from [35]; [^{3}H] cPD1 is used as reporter peptide in a competitive binding assay with the pheromone receptor TraC. [c] The prefix c or i indicates 'conjugation trigger' (pheromone) and inhibitor peptide, respectively.

transfer of the conjugative plasmid pPD1 of *E. faecalis*. The overall identity between TraC and PrgZ is 87%, and the N-terminal parts (residues 1 to 300 out of a total of 545 amino acids) are even 97% identical. In contrast to PrgZ, TraC does not use Opp to effect the uptake of the pheromone. In mutants strains lacking *oppD*, the uptake of cPD1 was comparable with the wild type. Most likely, TraC interacts with the membrane components of another ABC transporter ([35], *table III*).

Genome database searches of *E. faecalis* have revealed the genetic determinants for the pheromones, which had remained elusive for a long time. It appears that the peptides correspond to internal sequences of signal peptides of apparent lipoprotein precursors (*table III*). The role of the lipoprotein products, after processing of their signal sequences and related pheromones, is unknown. The finding that lipoproteins are the precursors of the pheromones supports the hypothesis that the membrane-associated protease Eep is involved in the production of the peptide ([9]; *table III*). The Eep protein (enhanced expression of pheromone) is a membrane-bound protease that stimulates the production of several pheromones by processing the prepheromone [5].

After transport of the pheromone into the donor cell, the *prgB* gene becomes induced, which results in the production of the aggregation substance Asc10. Asc10 contributes to the formation of a stable mating complex between donor and recipient cells and establishes an efficient transfer of the conjugative plasmid [6].

Buttaro and coworkers showed in cell fractionation studies that the majority of the pheromones produced are associated with the cell, primarily the cell wall fraction. The pheromones represented a mixture of mature and precursor peptide, suggesting that the processing of the pheromone takes place outside the cell. After transfer of the plasmid, the newly created donor cell continues to secrete the pheromone. There is, however, no self induction by this signal of endogenous origin, because the plasmid also carries the genes for two mechanisms that neutralize the activity of the produced pheromone. The first mechanism involves the production of an inhibitor peptide (*table III*), that is released in sufficient quantity to neutralize the activity of the pheromones released into the medium. The second mechanism concerns the expression of the *prgY* gene, which encodes a putative membrane protein. The PrgY protein lowers the amount of pheromone in the cell wall but does not affect the levels of secreted pheromone in the medium. It is not yet clear how PrgY achieves this neutralizing effect. It is possible that PrgY degrades the cell-associated pheromone, interferes with peptide binding to PrgZ, or acts by some other mechanism [7].

4.4. Chemotaxis

The Dpp (dipeptide permease) system of *E. coli* is not only involved in transport of dipeptides, but

it is also important for peptide taxis. In strains unable to transport dipeptides, but containing wild-type levels of the dipeptide binding protein (DppA), the chemotaxic response towards peptides is comparable to that in the wild type. This is consistent with the notion that only the dipeptide binding protein of Dpp is involved in chemotaxis [1]. Later research showed that at least five different receptors (methyl-accepting chemotaxis proteins (MCPs)) are present in the cytoplasmic membrane of enteric bacteria to recognize attractants. The interaction of the attractant is either directly with the receptor, that is, in the case of the serine, aspartate and citrate taxis, or via periplasmic binding proteins, that is, in the case of maltose, ribose, galactose, glucose and dipeptide taxis. In the latter case, the liganded receptors interact with the appropriate membrane receptor. These MCPs thus have to compete with the membrane components of ABC transporters for binding of periplasmic binding protein.

The membrane receptor transmits the chemical signal across the membrane, which triggers a complex communication pathway. This pathway involves CheA, a histidine kinase, CheY, a response regulator, CheW, a receptor coupling factor, and CheZ, an enhancer of Che-Y-P dephosphorylation. The CheA and CheY proteins are like a two-component regulator system but, in contrast to, for instance, ComE in *S. pneumoniae*, the CheY protein is not a transcription factor but rather a motor switch [34]. Activation of the chemotaxis pathway elicits a change in the flagellar rotation, that is, the flagellum changes from clockwise rotation, resulting in tumbling, into counterclockwise rotation. Consequently, the swimming becomes directional towards the attractant.

4.5. Virulence

Cationic antimicrobial peptides are important components of the innate defense of animals and plants against bacterial infections. The small peptides can adopt amphipathic α-helical structures and form voltage-gated channels in bacteria. The spectrum of activity of these peptides is broad and includes Gram-negative and Gram-positive bacteria, fungi, and parasites. The antimicrobial peptides tend to be found in parts of the organism that most likely come into contact with pathogenic bacteria, like skin, ear, eye, and epithelial surfaces [19].

Successful pathogenic bacteria have evolved mechanisms to withstand the pore-forming activity of these molecules. A facultative intracellular pathogen like *S. typhimurium* can replicate within macrophages and resist the battery of cationic peptides that are found within the lysosomal granules. To identify the determinants of the resistance to antimicrobial peptides, mutants of *S. typhimurium* have been screened that were hypersensitive to antimicrobial peptides. This revealed that the *sap* (sensitive to antimicrobial peptides) operon is necessary for resistance to protamine. This operon consists of five open reading frames: *sapA*, *sapB*, *sapC*, *sapD* and *sapF*, and the system exhibits similarity with the ABC transporter superfamily. The substrate binding protein SapA has highest identity with receptor proteins involved in peptide transport [39]. The role of the *sap* operon in virulence has recently been questioned, because *E. coli* with an increased K^+ uptake capacity are less sensitive to protamine irrespective of whether a function Sap system is present. This work has led to the hypothesis that protamine forms a channel through which K^+ leaves the cell, and that high K^+ influx can rescue the cell until the protamine is detoxified [52]. In addition to a possible role of Sap, other defense mechanisms have been implicated in resistance to antimicrobial peptides. It has been proposed that resistance to antimicrobial peptides involves lowering of the adherence of the peptides to the bacterial outer membrane. Changes in the outer membrane concerned the addition of aminoarabinose and palmitate to the lipid A moiety of lipopolysaccharide [16]. Another proposed resistance mechanism involves the upregulation of *pgtE* expression, which specifies an outer membrane endopeptidase that cleaves the peptides and thereby neutralizes their toxicity [15]. The resistance toantimicrobial peptides that form voltage-gated ion channels may thus depend on at least three different mechanisms: (i) changes in the bacterial outer membrane to prevent adherence of the peptides; (ii) elevated expression of the outer membrane endopeptidase PgtE to degrade the peptides; and (iii) transport of the antimicrobial peptides into the cell, where they are degraded by the intracellular proteolytic system.

5. Concluding remarks

The role of peptides in the environment of bacteria is twofold: (i) they can be used as a source of nitrogen or metabolic energy; and (ii) they can inform

the cell about the local environmental conditions. If peptides are used as a source of nitrogen or energy, it is necessary that the substrates be transported into the cell and that the system have broad specificity. The majority of peptide transport systems in bacteria are ABC transporters, but ion-linked uptake systems have been reported in a number of cases [18]. For the response towards peptides involved in signaling, microorganisms have developed two different strategies. In one mechanism the peptide is transported into the cell and functions intracellularly, whereas in the second mechanism the peptide binds to a receptor that transduces the signal across the membrane. The first mechanism involves ABC transporters and often a specific peptide binding protein, like PrgZ, that interacts with the membrane components of Opp. The second mechanism is ABC-independent and involves so-called two-component regulator systems. Besides a histidine kinase peptide receptor there is a response regulator involved that either up- or downregulates the expression of particular genes or switches on/off specific pathways such as those that control the flagellar motor.

The different signaling peptides are transported out of the cell in different ways. In the case where the peptides are produced as precursors with a typical signal sequence such as the competence stimulating factor (CSF) and PhrA, it is likely that the molecules are translocated across the cell membrane via the Sec machinery [27]. The peptides with an N-terminal double-glycine signal peptide, like the competence stimulating peptide in *S. pneumoniae*, are exported via dedicated ABC transporters with proteolytic activity to cleave off the signal sequence. This type of transport system is also known for peptide bacteriocins [37]. For mating pheromones in yeast it is known that the peptides are excreted via the Ste6P ABC transporter, a homologue of the multidrug resistance protein P glycoprotein. In yeast, there are also pheromones that are excreted in a Ste6p-independent way. These pheromones might be excreted by another ABC transporter, ion-coupled transporter, or possibly by diffusion [8].

A striking aspect of the binding protein-dependent peptide transport systems concerns the differences in selectivity. In some cases, the proteins (e.g., PrgZ and TraC) exhibit a very high binding affinity, the dissociation constants for peptides are in the nanomolar range. PrgZ and TraC are highly similar but they seem to be dedicated to one or a few specific hydrophobic peptides. On the other hand, the general binding proteins (OppA) of the Opp systems have dissociation constants in the micromolar range for a wide variety of peptides, differing in length and amino acid composition. This correlates perfectly with the function of these peptide binding proteins. OppA plays a role in nutrition and should be able to accept peptides irrespective of their sequence and composition, whereas the binding proteins involved in conjugation should only respond to a specific signal. However, until now there is little structural information that explains the differences in specificity.

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